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MOLECULAR PREVALENCE OF DIFFERENT GENOTYPES OF *THEILERIA ORIENTALIS* DETECTED FROM CATTLE AND WATER BUFFALOES IN THAILAND

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ABSTRACT: Here we report on an epidemiological study regarding the molecular prevalence of different genotypes of *Theileria orientalis* present among domestic cattle and water buffalo populations bred in Thailand. A phylogenetic analysis based on the parasitic gene encoding a major piroplasm surface protein revealed the presence of 5 genotypes (Types 1, 3, 5, 7, and N-3) in cattle and 7 genotypes (Types 1, 3, 4, 5, 7, N-2, and N-3) in water buffaloes. Types 4, 7, and N-3 of *T. orientalis* were reported for the first time in water buffaloes. The previously reported C and Thai types from Thailand clustered as types 7 and 6, respectively, in the present analysis. Great similarities were observed among nucleotide sequences of isolates of the same genotype from cattle and water buffaloes, and, therefore, water buffaloes were considered to serve as a reservoir for these genotypes of *T. orientalis* in Thailand. In conclusion, *T. orientalis* parasites circulating in Thailand are more diverse in their genetic characters than previously anticipated.

Theileria parasites that infect different livestock species are relegated into 2 groups. *Theileria parva* and *Theileria annulata* form the first group that causes malignant lymphoproliferative theileriosis in cattle (Onuma et al., 1997), while another group of species whose taxonomic classifications are still under debate causes a non-lymphoproliferative bovine theileriosis (Minami et al., 1980; Kim et al., 1998). This second group includes *Theileria sergenti/Theileria buffeli/Theileria orientalis* (Uilenberg et al., 1985; Fujisaki et al., 1994). The disease caused by the benign group (we used "*T. orientalis*" as the common name in the present study) has been often described as a subclinical condition, although the clinical picture may include anemia and other nonspecific signs (Kawazu et al., 1992). Nonetheless, the disease may eventually lead to severe economic losses in endemic areas (Minami et al., 1980; Baek et al., 1990; Kim et al., 2004). Studies conducted on *T. orientalis* in relation to diagnosis and prevention have generated valuable information regarding survival strategies adopted by these parasites against host immunity (Katzer et al., 2010).

Many bovine hemoprotozoan parasites are capable of developing an antigenic polymorphism to escape from host immune responses, which ensures their long-term survival (Katzer et al., 1994). Similar observations have also been made with *T. orientalis*; the antigenic polymorphism defined by a major piroplasm surface protein (MPSP) has been extensively investigated (Zuang et al., 1994; Kubato et al., 1996).

MPSP gene sequences derived from different isolates of *T. orientalis* form a number of clusters in phylogenetic analyses, and, importantly, these clusters have shown different antigenic properties (Jeong et al., 2010). These findings have led researchers to conclude that the *T. orientalis* group can be classified with regard to any number of different genotypes (Kim et al., 1998; Ota et al., 2009; Yokoyama et al., 2011). Initially, 4 genotypes (I, C, B, and Thai types) were described for the parasites (Kakuda et al., 1998; Sarataphan et al., 1999, 2003). Kim et al. (1998) suggested that the benign *Theileria* species possessed 6 genotypes (Types 1–6). In addition, types 7 and 8 were subsequently identified (Kim et al., 2004; Ota et al., 2009; Jeong et al., 2010). Interestingly, analyses of *MPSP* gene sequences of Vietnamese isolates indicated the presence of additional genotypes that included types N-1, N-2, and N-3 from sheep, water buffalo, and cattle, respectively (Kawasu et al., 1999; Khukhuu et al., 2011). Our recent study on Mongolian bovine isolates also generated a range of *MPSP* gene sequences, with many of them resembling the novel type N-3 of *T. orientalis* (Altangerel et al., 2011).

In contrast to the above classification, Bai et al. (2002) and Liu et al. (2010) have described type 6 as *T. sinensis*, and argued that it may be a different species based on the molecular characters and transmission vectors. Similar results were obtained in a recent study on *T. orientalis* conducted in China, where the type 6 was not detected among the sampled populations (Liu et al., 2011).

Previously, Kakuda et al. (1998) indicated that the *T. orientalis* isolates collected in Thailand were phylogenetically separated from those of China and the United States. In addition, the presence of Thai, B, and C types in the Thailand cattle population was reported by Sarataphan et al. (1999). Another study using the DNA samples extracted from blood of 214 cattle and 33 water buffaloes concluded that the C and Thai types of *T. orientalis* were circulating among these animal populations in Thailand (Sarataphan et al., 2003). Because the C type detected in Thailand was indeed closer to Indonesian isolates than the Chitose type of Japanese isolates, the authors suggested that the C type in Thailand must belong to a different genotype of *T. orientalis*. Since the latter study was based on an allele-specific PCR method and few gene sequences, the findings and descriptions were limited to the existing genotypes. Therefore, we conducted the present investigation, focusing on the genetic diversity of *T. orientalis* in Thai isolates using greater numbers of the newly determined *MPSP* gene sequences based on the current criteria for genotyping.

MATERIALS AND METHODS

Sample collections

Blood samples of cattle were collected from 3 locations, including Chiang Rai, Chiang Mai, and Lampang located in the northwest region of Thailand, in September 2009. Five locations, including Roi Et, Ubon-Ratachathani, Si Sa Ket, Surin, and Buri Ram, were selected from the northeast region for sample collection from water buffaloes in January 2010 (Fig. 1) (Terkawi et al., 2011). Approximately 1.5 ml of whole blood

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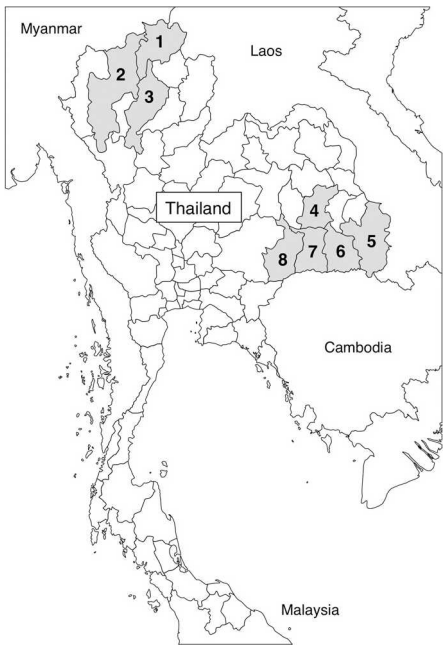

FIGURE 1. Locations of the areas studied in Thailand. The regions: 1, Chiang Rai; 2, Chiang Mai; 3, Lampang; 4, Roi Et; 5, Ubon Ratachathani; 6, Si Sa Ket; 7, Surin; 8, Buri Ram.

was collected from each animal and transferred into sterile tubes containing EDTA (NIPRO, Osaka, Japan). The blood samples were collected from 200 cattle and 255 water buffaloes from the selected locations.

DNA extraction

Two hundred microliters of the whole blood were subjected to a DNA extraction protocol. A Qiagen blood DNA extraction kit (Qiagen, Hilden, Germany) was employed, according to the manufacturer's instructions. Finally, the DNA sample was prepared in 100 µl of the elution buffer that was provided with the kit. Extracted DNA samples were stored at −30 C until use (Ota et al., 2009).

Polymerase chain reaction, cloning, and sequencing

A previously described set of forward (5′-CTTTGCCTAGGATACTTCCT-3′) and reverse (5′-ACGGCAAGTGGTGAGAACT-3′) primers was used for the PCR detection of *T. orientalis MPSP* genes (Ota et al., 2009). The reaction mixture of 10 µl, which contained 0.1 µl of each primer from 10 µM stocks, 5 µl of 2 × Ampdirect plus (Shimadzu Biotech., Kyoto, Japan), 0.1 µl of Extaq DNA polymerase (Takara, Tokyo, Japan), 3.7 µl of double distilled water (DDW), and 1 µl of the template DNA, was amplified under previously described thermal conditions (Ota et al., 2009). Briefly, an activation step at 94 C for 10 min was followed by 35 cycles that each consisted of a denature step at 94 C for 1 min, an annealing step at 58 C for 1 min, and an extension at 72 C for 1 min. After the final extension at 72 C for 4 min, positive reactions were identified by detecting a 776-bp specific band on agarose gels after electrophoresis.

Selected PCR-positive samples from each location were then amplified under conditions similar to the screening PCR, except for the composition of the reaction mixture. The total volume for each reaction was increased to 50 µl, which included 1 µl of the DNA sample, 10 µl of Expand HiFi Plus reaction buffer, 1 µl of each 10 µM primer, 0.5 µl of Taq DNA polymerase (Expand HiFi Plus; Roche Applied Science, Basel, Switzerland), 1 µl of 10 mM Nucleotide Mix (Roche Applied Science), and 35.5 µl DDW. The amplified PCR products were gel-extracted and then ligated into a TA-cloning plasmid vector (PCR 2.1-TOPO; Invitrogen, Carlsbad, California). Plasmids with the inserted DNA fragment were then transformed into *E. coli* competent cells (TOP 10; Invitrogen) and cloned. After overnight incubation, clones were isolated and cultured at 37 C. The plasmids were then extracted from the cultures using a commercial QIAprep Spin Miniprep kit (Qiagen), and the presence of the inserted DNA fragment was confirmed by the PCR method as described above. Finally, the nucleotide sequences of the DNA fragments were determined according to a previously described protocol (Yokoyama et al., 2011).

Phylogenetic analysis

The nucleotide sequences obtained in the present study, together with the previously registered *MPSP* gene sequences in GenBank, were used to construct a phylogenetic tree as described by Khukhuu et al. (2011). In brief, the GENTYX 7.0 software package was used to analyze the nucleotide sequences (GENTYX, Tokyo, Japan). Furthermore, a ClustalW program (Thompson et al., 1994) was used to construct the guide tree based on the multiple alignment and neighbor joining methods (Perrière et al., 1996). The confidence of branching pattern of the constructed tree was estimated by a Bootstrap test (Felsenstein, 1985).

RESULTS

The results of screening MPSP-PCR assay showed the presence of *T. orientalis* parasites circulating in the blood of cattle and water buffaloes bred in the 8 regions of Thailand (Fig. 1). Approximately 25% of the cattle populations in Chiang Rai and Chiang Mai regions were positive for *T. orientalis* infections, while relatively high numbers of positive animals (50%) were found in the cattle population of the Lampang area (Table I). However, none of the water buffaloes from the Ubon-Ratachathani region were positive in the MPSP-PCR assay, whereas buffaloes in the Roi Et region exhibited the highest positive percentages (25.6%) (Table I). The average percentages of positive animals in the studied areas were 31.5 and 9.4% for the cattle and water buffaloes, respectively.

The *MPSP* gene sequences of *T. orientalis* isolates derived from the cattle (n = 28) and water buffaloes (n = 24) were phylogenetically analyzed to identify the genotypes of *T. orientalis* circulating in the domestic animals of Thailand (Fig. 2). Five genotypes (Types 1, 3, 5, 7, and N-3) and 7 genotypes (Types 1, 3,

TABLE I. MPSP-PCR diagnosis and *MPSP* gene genotyping for *Theileria orientalis* in cattle and water buffaloes collected in Thailand.

Sample origin	Animal	No. total collected samples	No. positive samples (%)	Isolated genotypes (No.)
Chiang Rai	Cattle	96	24 (25.0)	1 (1), 3 (2), 7 (3), N-3 (2)
Chiang Mai	Cattle	54	14 (25.9)	1 (8), 3 (2)
Lampang	Cattle	50	25 (50.0)	3 (1), 5 (4), 7 (5)
Roi Et	Water buffalo	49	13 (26.5)	1 (3), 3 (1), 4 (1), 5 (1), 7 (4), N-2 (1), N-3 (2)
Buri Ram	Water buffalo	70	5 (7.1)	1 (2), 7 (3)
Surin	Water buffalo	35	4 (11.4)	5 (1), 7 (3)
Si Sa Ket	Water buffalo	48	2 (4.2)	7 (2)
Ubon-Ratachathani	Water buffalo	53	0 (0.0)	

FIGURE 2. A phylogenetic tree of the *MPSP* gene sequences derived from blood samples of cattle and water buffaloes in Thailand (52 sequences), together with previously registered sequences from the GenBank database (61 sequences). The *MPSP* gene sequences determined in the present study are shown in boldfaced type and refer to the GenBank accession numbers as indicated at the end of each branch. Numbers shown at branch nodes indicate bootstrap values. Recently a type-6 group was classified as *T. sinensis* (Liu et al., 2010).

4, 5, 7, N-2, and N-3) were identified from cattle and water buffalo populations, respectively (Table I). Among the 5 types detected from the cattle population, type 3 was found in all 3 regions surveyed in the present study, while types 1 and 7 were detected from 2 locations (Chiang Rai and Lampang). Type 5 was found only in Lampang and the N-3 was detected only from Chiang Rai (Table I). The cattle population in the Chiang Rai region harbored 4 different genotypes (Types 1, 3, 7, and N-3), while the cattle in the Chiang Mai region were infected with only 2 different genotypes (Types 1 and 3) (Table I).

Among the water buffalo populations bred in the regions of Thailand selected in this study (Fig. 1), 7 genotypes (Types 1, 3, 4, 5, 7, N-2, and N-3) of *T. orientalis* were identified (Table I). Type 7 was predominantly detected in all areas where *T. orientalis*–positive

buffaloes were found. The highest genotypic variation was found among *T. orientalis* isolates in the population of the Roi Et region, where all 7 genotypes identified in the current study were present (Table I). The previously reported C type (AB081329) and Thai type (AB010703) from Thailand were clustered as types 7 and 6, respectively, in the phylogenetic analysis (Fig. 2).

DISCUSSION

The *MPSP* gene has been well recognized as an epidemiological molecular marker for understanding the genetic diversity of *T. orientalis* (Kawazu et al., 1992, 1999; Kakuda et al., 1998; Sako et al., 1999; Sarataphan et al., 1999; Ko et al., 2008; Ota et al., 2009). The multiple alignments of *MPSP* genes derived from different isolates indicate the presence of several clusters with different antigenic characters, and thus researchers have described them as different genotypes (Kim et al., 2004; Jeong et al., 2010; Yokoyama et al., 2011). Previously, the identification of *T. orientalis* genotypes has been based on the geographical locations of the isolates. However, further studies have proved the lack of relationship between the genotypes and the geographical locations (Jeong et al., 2010). Therefore, it would be more appropriate to adopt the numerical classification method proposed by Kim et al. (1998).

Initially, there were 6 genotypes (Types 1–6) of *T. orientalis* (Kim et al., 1998), and, subsequently, 2 additional genotypes (Types 7 and 8) were added (Jeong et al., 2010). In our recent study, we described 3 more genotypes (Types N-1, N-2, and N-3), based on phylogeny with previously reported *MPSP* gene sequences and those recorded in Vietnamese *T. orientalis* (Khukhuu et al., 2011).

The present study revealed the presence in Thailand of at least 5 and 7 genotypes in cattle and water buffaloes, respectively, and that type 3 was detected in cattle populations from all the selected locations. One of the recently added genotypes, type N-3, was also detected in cattle from the Chiang Rai region. This finding is in agreement with our recent investigation on the molecular epidemiology of *T. orientalis* from Mongolian cattle (Altangerel et al., 2011). In water buffaloes, type 7 of *T. orientalis*, which was isolated from 4 of 5 locations, was found to be the dominant genotype in Thailand. In addition, types N-2 and N-3 were also identified in the water buffaloes from Roi Et region. The existence of types 4, 7, and N-3 reported here is the first report of these forms in water buffaloes.

In the present study, the previously described C and Thai types were classified as types 7 and 6, respectively, using the present criteria. The phylogenetic analysis revealed that the Indonesian isolates (AF102500) also belong to type 7, together with Thai isolates originally described as C type (AB081329), whereas the Japanese C types (D12689) are clustered with type 1 (Fig. 2). Although the cattle and water buffalo samples were collected from different locations, the sequences of *MPSP* genes derived from both animals showed great similarities within particular genotypes. This finding appear to support the previous suggestion that water buffaloes may serve as a reservoir for many hemoprotozoan parasites (Oura et al., 2010).

Although a previous study detected the B type (type 3) among cattle population in Thailand (Sarataphan et al., 1999), a subsequent epidemiological survey by the same investigator failed to demonstrate the presence of B type (Sarataphan et al. 2003). However, in the present study, type 3 was identified in cattle populations from all locations. In addition, type 2 (I type) of *T. orientalis*, which has been reported in Japan, the Republic of Korea, and China (Kakuda et al., 1998; Kim et al., 1998; Jeong et al., 2009; Ota et al., 2009; Yokoyama et al., 2011), was not detected in the current study. This finding is in agreement with the previous observation made by Sarataphan et al. (1999, 2003).

A previous study had identified that the Thai type, which is clustered under type 6 in the present study, was the dominant genotype in Thailand (Kakuda et al., 1998; Sarataphan et al., 1999). However, none of the sequences was classified as type 6 in the present study. This finding may support the previous assumption that described type 6 as *T. sinensis* (Bai et al., 2002; Liu et al., 2010). The results of the pairwise comparisons revealed very low homologies between Thai type (AB010703) and other reported sequences clustered within type 6 (less than 90%). Therefore, the Thai type may form a separate branch from other registered sequences within this group (Fig. 2). Sarataphan et al. (2003) found that about 86 and 60% of cattle and water buffaloes, respectively, were positive for benign *Theileria* parasites in Thailand. In contrast, the present study indicates that only 31.5 and 9.4% were positive for parasite infections in the cattle and water buffalo populations, respectively. The observed differences may be due to the presence of Thai type, which could be considered as *T. sinensis*.

Although we were unable to detect the genotypes 2, 6, 8, and N-1 among cattle and water buffalo populations in Thailand, these findings may not be conclusive, as the blood samples were collected only from selected locations. In addition, the level of parasitemia might be below the detection limit of the PCR technique. Therefore, a large-scale epidemiological study is essential to confirm and expand the findings of the current investigation.

Consequently, we were able to identify several new genotypes of *T. orientalis*, which had not been described previously in Thailand. This indicates that *T. orientalis* parasites circulating in Thai cattle and water buffaloes have a much higher genetic diversity than previously expected.

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